ANTIMICROBIAL ACTIVITIES OF *VERNONIA AMYGDALINA, OCIMUM GRATISSIMUM AND GARCINIA KOLA*

Nworah, I.I. * and Umeaku, C.N.

Department of Microbiology,
Chukwuemeka Odumegwu Ojukwu University, Uli
Anambra State Nigeria .

ABSTRACT

This work was undertaken to investigate the antimicrobial activities of *Ocimum gratissimum*(Scent leaf), *Vernonia amygdalina*(Bitter leaf) and *Garcinia kola*(kola Bitter) *leaves and seeds* extracts against pathogenic *Escherichia coli, Staphylococcus aureus* and *Candida albicans.* The phytochemical analysis of the leaves and seeds extracts of the plants were determined qualitatively and quantitatively using chemical and spectrophotometric methods. The inhibitory activity was carried out using agar well diffusion method. Tube dilution technique using double – fold serial dilution method was employed for assaying the Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) at various concentrations (31.25, 62.5, 125, 250 and 500 mg/ml). The pathogenicity study was carried out by infecting the mice with the test organisms, monitoring the infected mice and examination of visceral organs of the infected mice for pathological changes. The phytochemical analysis revealed the presence of alkaloids, tannins, flavonoids, saponins, phenolics, glycosides and steroids. The study showed that the activities of ethanolic extracts of *Ocimum gratissimum*(Scent leaf), *Garcinia kola*(Bitter kola) and *Vernonia amygdalina*(Bitter leaf) against *Staphylococcus aureus, Escherichia coli* and *Candida albicans* were significantly higher ($P<0.05$) than that of the Petroleum ether and aqueous extracts of *Ocimum gratissimum*(Scent leaf), *Garcinia kola*(Bitter kola) and *Vernonia amygdalina* (Bitter leaf). The inhibitory activities of *Ocimum gratissimum* extracts were most significant ($P<0.05$) against *Staphylococcus aureus* and non-significant ($P>0.05$) against *Eschericia coli*. Effect of *Vernonia amygdgalina* extract on *Candida albicans* was not significant ($P<0.05$).The results of the Minimum Inhibitory Concentrations (MICs) and Minimum Lethal Concentrations (MLCs), showed pronounced activities of which ethanolic extracts showed most activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. The histopathological study of the viscera organs of the infected mice revealed obvious pathological signs. The study showed that the leaves and seeds extracts of the studied plants possess antimicrobial properties and could serve as alternative therapy for infections caused by the test organisms.

INTRODUCTION

The search for current sources of antibiotics in a global challenge pre-occupying research instruction, pharmaceutical companies and the academic, since many infection agents are becoming resistant to synthetic drugs [(1)].The use of medicinal plants as a source for relief from illness can be traced back from several millinia, to written documents of the early civilization [(2)].Many of the drug currently used to treat bacterial and other infection were first isolated from natural sources, including ethnomedicinal plants [(3)].The use of plants in traditional medicine contain array of substances that can be used to treat chronic and infectious diseases.

Among the estimated 250,000 – 500,000 plants species only a small percentage have been investigated phytochemically and the fraction submitted to biological or pharmacological screening is even smaller [(4)] . The World Health Organization (WHO) has reported that about 80% of the world's population is depending on traditional medicine for treatment of such diseases as diarrhea, cough, fever, headache etc [(5)] . In some parts of Africa, for example Nigeria, Kenya etc, extracts of *Vernonia amygdalina* have been found to possess antimicrobial activity against *Plasmodium* and other sexually transmitted diseases [(6)] . also Garcinia kola have been successfully used in the treatment of diseases such as diabetes, throat infection, gonorrhea etc. *Ocimum gratissimum* have been used by many traditional healers in Nigeria to treat such ailments as stomach disorders, feverish condition, cough and hypertension [(7)] . Therefore, the objective of the research is to evaluate the antimicrobial activities of *Vernonia amygdalina* (bitter leaf), *Ocimum gratissimum* (scent leaf) and *Garcinia kola* (bitter kola) against *Staphylococcus aureus , Escherichia coli* and *Candida albicans.*

MATERIALS AND METHODS

SOURCE OF PLANT SAMPLES

The fresh leaves of *Vernonia amygdalina, Ocimum gratissimum* and the seeds of *Garcinia kola* were collected at Ozubulu in Ekwusigo LGA of Anambra State and were identified by Dr. Garuba Omosun of Botany Department, Michael Okpara University of Agriculture, Umudike, Umuahia, Abia State.

PREPARATION OF SAMPLES FOR EXTRACTION

The leaves of both *Vernonia amygdalina* and *Ocimum gratissimum* were plucked off and the seeds of *Garcinia kola* removed from the fruit. They were thoroughly washed with distilled water and kept at room temperature for 14 days to dry. The dried samples were ground with sterile electric blender, weighed and kept ready for extraction of active ingredient [(8)] .

EXTRACTION PROCEDURE

A 20 g portion of each of the samples was extracted by solvent extraction in 200 ml of ethanol, Petroleum ether and water respectively for 3 days. The resulting extracts were filtered using Whatman No 1 filter paper. The extracts were evaporated to dryness at room temperature in a steady current [(9).]

CONCENTRATION OF THE EXTRACTS

One hundred milliliters (100ml) of ethanol and petroleum ether extracts where exposed at room temperature for 7 days. While that of the aqueous extract was concentrated using electric oven for 5 days. The concentrates were used to prepare the actual concentration of the extracts (500mg/ml), used for the analysis.

DETERMINATION OF THE EXTRACTIVE VALUE

The determination of the plants extractive values were carried out to get the exact weight of the extracts in the prepared concentrations which were used for the analysis. One gram (1.0g) of each of the extracts was evaporated in an evaporating dish of known weight in an oven to dryness at 30^{o}C and weighed. The dish containing the residue was allowed to cool and then reweighed. The weight of the residue was obtained by subtracting the initial weight of the empty dish from the weight of the dish and residue. This processes were repeated two (2) times [(10)] .

PHYTOCHEMICAL ANALYSIS

The extracts obtained were subjected to qualitative and quantitative phytochemical screening [(11)], to determine the presence of bioactive agents such as alkaloids, saponins, tannins, glyceroids, flavonoids, steroids and phenolics.

ISOLATION AND IDENTIFICATION OF TEST ORGANISMS

The urine sample of female patients with urinary tract infection (UTI) was collected with sterilized container from the Microbiology Department of Nnamdi Azikiwe University Teaching Hospital, Nnewi. The sample was aseptically inoculated into petri dishes containing Eosine Methylene Blue (EMB), Manitol salt agar and Sabaurand Dextose Agar (SDA) respectively, using pour plating technique as described by Iheukwumere and Umedum(2013). The plates were incubated inverted at 37^{o}C for 24 h. After 24 h, the growth on the three plates were subcultured asceptically into the three media mentioned above, using the stricking method as described by [(12)]. The pure cultures generated were characterized and identified using their colonial descriptions; gram staining, microscopy and biochemical reaction [(13)].

MAINTENANCE OF TEST ORGANISMS

The isolated organisms were aseptically subcultured into Nutrient broth and Sabaurand Dextose broth. They were incubated at 37^{o}C for 24 h. The 24 h cultures were used for antimicrobial activities.

PREPARATION OF STANDARD ANTIBIOTICS

The standard antibiotics used for this work were Ciprofloxacin and Nystatin. This was because Ciprofloxacin is effective for the treatment of bacterial infections whereas Nystatin is used for the treatment of fungal infections especially Candidiasis. 500 mg / ml of standard antibiotic was prepared by dissolving 5 g in 10 ml of distilled water [(14)].

SENSITIVITY TESTING USING AGAR WELL DIFFUSION

The 24 h broth culture of both bacterial (*Escherichia coli, Staphylococcus aureus*) and fungal (*Candida albicans*) test organisms were aseptically inoculated into Muller Hinton Agar and Sabaurand Dextose agar respectively [(15)]. A 5 mm cock borer was used to make wells on the solidified cultures. 0.1 ml of each of the extracts and antibiotics were added in each of the wells for each organism. The plates were incubated vertically at 37^{o}C for 24 h. The diameter of the zones of inhibition of each extract and standard antibiotics against the test organisms were measured and recorded.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The Minimum Inhibitory Concentration (MIC) was carried out using tube Dilution Technique of Iheukwumere and Umedum (2013). Different concentrations of the extracts were obtained using double fold serial dilution method. 1 ml of each of the test organisms was added into the test tubes and incubated at 37^{o}C for 24 h. The MIC was recorded as the lowest concentration (i.e. the highest dilution) of the extract that inhibited the growth of the test organism [(16)].

DETERMINATION OF THE MINIMUM LETHAL

CONCENTRATION (MLC)

The Minimum Lethal Concentration was determined through the modified methods of Iheukwumere and Ubajekwe (2012). Equal volumes of various concentrations that did not produce any visible growth from MIC was subcultured into Nutrient agar for bacteria, and Sabaurand Dextose agar for fungi and incubated at 37^{o}C for 24 h. The MLC was recorded as the minimum concentration of extracts that killed the test organism within the specific time.

RESULTS

The characteristic properties of the test organisms used for this study are shown in Tables 1 and 2. The test organisms; *Staphylococcus aureus, Escherichia coli* and *Candida albicans* were isolated from the urine sample of a patient with urinary tract infections (UTI). The *Candida albicans* was characterized and identified using its macroscopic appearance, microscopic characteristics, biochemical reaction and with the aid of fungal atlas. The bacterial test organisms were characterized and identified based on their colonial description, gram reactions and biochemical reactions.

Table 1: Characteristics and Identities of the test bacteria

Parameter	*Staphylococcus aureus*	*Escherichia coli*
Appearance on agar plate	Golden yellow	Green metallic sheen
Margin	Entire	Entire
Gram reaction	+	-
Shape	Coccus	Rod
Catalase test	+	+
Motility test	-	+
Indole test	-	+
Coagulase test	+	-
MR test	-	+
VP test	-	-
Glucose	A/G	A/G
Maltose	A	A/G
Lactose	A/G	A/G

+	=	Positive	A	=	Acid
-	=	Negative	G	=	Gas

Table 2: Characteristics and identity of the test fungus

Parameter		*Candida albicans*
Macroscopic	Observation	They were soft cream coloured colonies with a yeasty odour.
Microscopic	Observation	The colony produced pseudohyphae and blastospores when the colony was inoculated in a human serum and incubated at 35^{o}C for 3h, there was formation of germ tube.

The qualitative and quantitative phytochemical determination of the leaves extracts are showed in Tables 3 and 4. The analysis revealed the presence of alkaloids, tannins, flavonoid, saponins, phenolics, glycosides and steroid in the three plants; *Garcinia kola*; *Ocimum gratissimum* and *Vernonia amygdalina*. The study showed that *Ocimum gratissimum* contained high tannins with low saponins, steroids and glycosides. *Garcinia kola* contained low steroids while *Vernonia amygdalina* contained low flavonoids and phenolics.

Table 3: Qualitative phytochemical screening of the leaves extracts

Phytochemicals	*Garcinia kola*	*Ocimum gratissimum*	*Vernonia amygdalina*
Alkaloids	++	++	++
Tannins	++	+++	++
Flavonoids	++	++	+
Saponins	++	+	++
Phenolics	++	++	+
Glycosides	++	+	++
Steroids	+	+	++

+++ = High content ++ = Moderate content
+ = Low content - = Nil

Table 4: Quantitative phytochemical characteristics of the leaves extract (mg/g)

Phytochemcial	*Garcinia kola*	*Ocimum gratissimum*	*Vernonia Amygdalina*
Alkaloids	28.13	24.82	26.80
Tannins	7.61	8.88	7.31
Flaronoids	6.88	6.98	4.46
Saponins	5.64	3.31	6.50
Phenolics	4.93	4.26	2.70
Glycosides	2.64	1.41	2.96
Steroids	1.04	0.09	1.96

The diameter of zones of inhibitions of ethanolic, petroleum ether and aqueous leave extracts of *Ocimum gratissimum, Vernonia amygdalina* and *Garcinia kola* against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* are shown in table 5.

Table 5: Diameter of zones of inhibition of the inhibitory substances against the test organisms using 5mm Cork borer.

Substance	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*
EEG	9.40 ± 0.16	11.00 ± 0.82	6.70 ± 0.16
EEO	10.22 ± 0.34	11.60 ± 0.11	6.43 ± 0.22
EEV	9.83 ± 0.22	9.91 ± 0.37	7.10 ± 0.82
PEG	6.40 ± 0.16	7.23 ± 0.22	6.10 ± 0.82
PEO	7.33 ± 0.22	7.90 ± 0.82	5.70 ± 0.16
PEV	7.13 ± 0.22	7.70 ± 0.16	6.30 ± 0.82
AEG	-	5.90 ± 0.16	-
AEO	-	6.20 ± 0.82	-
AEV	-	5.83 ± 0.22	-
CPX	16.40 ± 0.16	22.10 ± 0.82	-
Nystatin	-	-	11.20 ± 0.82
Absolute Ethanol (0.1ml)	-	-	-
Petroleum Ether (0.1ml)	-	-	-
Distilled water (0.1ml)	-	-	-

EEG = Ethanolic extract of *Garcinia kola*
EEO = Ethanolic extract of *Ocimum gratissimum*
EEV = Ethanolic extract of *Vernonia amygdalin*
PEG = Petroleum ether extract of *Garcinia kola*
PEO = Petroleum ether extract *Ocimum gratissimum*
PEV = Petroleum ether extract *Vernonia amygdalina*
AEG = Aqueous of *Garcinia kola*
AEO = Aqueous extract of *Ocimum gratissimum*
AEV = Aqueous extract of *Vernonia amygdalina*
CPX = Ciprofloxacin

The study revealed that the extracts inhibited *Escherichia coli* most while *Candida albicans* was inhibited least. The results also showed that ethanolic leaves extracts exhibited the highest activity when compared to petroleum ether and acqueous leaves extract, while aqueous leaves extracts exhibited the lowest activity. *Ocimum gratissimum* leaves extracts exhibited the highest activity against *Staphylococcus aureus, Escherichia coli* and the least activity against *Candida albicans. Vernonia amygdalina* leaves extracts exhibited the highest activity against *Candida albicans* when compared to *Ocimum gratissimum* and *Garcinia kola.*

The minimum inhibitory concentrations (MIC) of the three studied plants against the tested organisms are shown in table 4.6 below.

Table 6: Minimum Inhibitory Concentration (MIC) of the inhibitory substances (mg /ml)

Inhibitory Substance	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*
EEG	500	250	500
EEO	250	250	500
EEV	500	250	500
PEG	500	500	500
PEO	500	500	500
PEV	500	500	500
AEG	-	-	-
AEO	-	500	-
AEV	-	-	-
CPX	125	62.5	-
Nystatin	-	-	250

EEG = Ethanolic extract of *Garcinia kola*

EEO = Ethanolic extract of *Ocimum gratissimum*

EEV = Ethanolic extract of *Vernonia amygdalin*

PEG = Petroleum ether extract of *Garcinia kola*

PEO = Petroleum ether extract *Ocimum gratissimum*

PEV = Petroleum ether extract *Vernonia amygdalina*

AEG = Aqueous of *Garcinia kola*

AEO = Aqueous extract of *Ocimum gratissimum*

AEV = Aqueous extract of *Vernonia amygdalina*

CPX = Ciprofloxacin

The minimum lethal concentrations (MLC) of the three studied plants against the tested organisms are shown in table 7 below.

Table 7: Minimum Lethal Concentration (MLC) of the inhibitory substances (mg / ml)

Inhibitory substance	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*
EEG	-	500	-
EEO	-	500	-
EEV	-	500	-
PEG	-	-	-
PEO	-	500	-
PEV	-	-	-
AEG	-	-	-
AEO	-	-	-
AEV	-	-	-
CPX	250	125	-
Nystatin	-	-	500

EEG = Ethanolic extract of *Garcinia kola*

EEO = Ethanolic extract of *Ocimum gratissimum*

EEV = Ethanolic extract of *Vernonia amygdalin*

PEG = Petroleum ether extract of *Garcinia kola*

PEO = Petroleum ether extract *Ocimum gratissimum*

PEV = Petroleum ether extract *Vernonia amygdalina*

AEG = Aqueous of *Garcinia kola*

AEO = Aqueous extract of *Ocimum gratissimum*

AEV = Aqueous extract of *Vernonia amygdalina*

CPX = Ciprofloxacin

The result of this study revealed that ethanolic, petroleum ether and acqueous leaves extracts of the three studied plants showed pronounced activity against the tested organisms; and the inhibitory effects among the leaves extracts differed significantly ($P \leq 0.05$) from the control (Ciprofloxacin and Nystatin). The study further revealed that the ethanolic leaves extracts of the studied plants exhibited most pronounced activity than the petroleum ether and aqueous leaves extracts when comparing the Minimum Inhibitory Concentration (MICs) and Minimum Lethal Concentrations (MLCs) as shown in Tables 6 and 7. The results showed that *Ocimum gratissimum* exhibited the highest activity and the activity was most on *Escherichia coli*.

DISCUSSION

About 80% of the population in Africa depend on traditional medicine for primary health care [17]. *Ocinum gratissinum, Vernonia amygdalina* and *Garcinia kola* are among the most useful plants for traditional medicine in Africa. Different parts of these plants extracted with different types of solvents have been used by researchers for investigating their properties. In this study, water ethanol and petroleum ether were used as the extractive solvents in deterring the antimicrobial activities of these plants. The phytochemcial components persent in the ethanol, petroleum ether and aqueous extracts were qualitatively and quantitatively determined. The phytochemicals found to be present include: steroids, tannins, saponin, phenolics alkaloids, flavonoids, glycosides. These phytochemicals were responsible for the antimicrobial effects of the leaves/seeds extracts on the test organisms [18]. It was observed that the activities of the ethanolic extracts were highest.

It was observed that the activities of the ethnolic extracts were highest while aqueous extracts were the least. This indicates that the active constituents of the plants have more ability to dissolve in ethanol, followed by petroleum ether and least in aqueous extracts as used in the study. Similar conclusion were drawn by other researchers [19] . The present research work suggest that organic solvent extraction is suitable to verify antimicrobial activities of medicinal plants and the findings were supported by any researchers [20].

The results of the Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of the leaves/seeds extract showed that the ethanolic and petroleum ether extracts possess reasonable antimicrobial activity against *Escherichia coli, Staphylococcus aureaus* and *Candida albicans,* while the activity of the aqueous extracts was not pronounced, except for the aqueous extract of *Ocimum gratissimum* exhibited the highest activity and the activity was most on Escherichia coli. Further research involving *in vivo* assays will be needed to establish the relationship between the MICs and MLCs obtained in this study and the effective doses that should be administered in ethno-medical practice. [21].

CONCULSION AND RECOMMENDATION

The study revealed that *Ocimum gratissimum, Garcina kola* and Vernonia amygdalina leaves extracts exhibited pronounced antimicrobial activities against the test pathogenic organisms; *Escherichia coli, Staphylococcus aureaus* and *Candida albicans.* Therefore, it is recommended that further studies involving in *vivo* assays will be carried out to establish the relationship between the MICs and MLCs obtained in this study and the effective doses that should be administered in ethno-medical practice.

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